2009 Annual Report

Grant No. N000140710076

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Performing Organization:

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Grant Title:
Green Synthesis of Phloroglucinol: Exploiting Pseudomonas fluorescens and Scale-Up

Grant Period:
October 04, 2006 through October 14, 2009

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
2009	**Annual**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
2009 Annual Report - Green Synthesis of Phloroglucinol: Exploiting Pseudomonas fluorescens and Scale]Up	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Draths Corporation 2367 Science Parkway, Ste. 2 Okemos, MI 48864**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**7**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

Technical Report

a. Scientific and Technical Objectives

A new generation of phloroglucinol synthesizing microbe has been evaluated using resin-based extractive fermentation at 1 L scale. Strategies to understand *in vivo* phloroglucinol synthase expression in this genetically engineered *E. coli* were examined. Improving phloroglucinol synthase activity is essential to increase the microbial phloroglucinol synthesis titer and yield. Strategies to identify novel phloroglucinol synthases were therefore explored. It is also believed that expression of phloroglucinol synthase *phlD* gene inside its native *Pseudomonas fluorescens* Pf-5 will likely deliever higher expression level and activity of this gene product. Constructing a phloroglucinol synthesizing *P. fluorescens* was therefore pursued.

b. Approach

Coupling metabolic engineering and reaction engineering, improved phloroglucinol synthesizing *E. coli* PG1/pKIT10.080 was evaluated under resin-based extractive fermentor-controlled conditions. Heterologous expression of *P. fluorescens* Pf-5 *phlD* gene in *E. coli* presents numerous complications. 2D protein gel electrophoresis and tandem mass spectroscopy were used to examine the relatively short catalytic lifetime and low activity of PhlD. In an attempt to identify better phloroglucinol synthases, the genetic diversity of *phlD* was explored. Bioinformatics analysis of potential *phlD* candidates in various organisms with significant amino acid sequence identity to our currently used Pf-5 *phlD* were examined. In parallel with these efforts, microbial synthesis of phloroglucinol in *P. fluorescens* was examined. Defined minimal salt medium was formulated to enable high density cultivation of *P. fluorescens* Pf1.162/pJA2.232 in the fermentor.

c. Concise Accomplishments

Under optimized resin-based extractive fermentation, *E. coli* PG1/pKIT10.080 synthesized 25 g/L of phloroglucinol. Coupling 2D protein gel electrophoresis and tendem mass spectroscopy, it was determined that heterologously expressing PhlD-encoded phloroglucinol synthase under fermonetor-controlled conditions led to significant formation of inclusion bodies. In attempts to identify phloroglucinol synthase alternatives using bioinformatics approach, five active phloroglucinol synthases were identified. High-cell density cultivation of *P. fluorescens* was achieved in fermentation vessels using minimal salts medium. Phloroglucinol production was observed by culturing *P. fluorescens* Pf1.162/pJA2.232 under defined fermentor-controlled conditions.

d. Expanded Accomplishments

The relative amount of soluble PhlD protein produced *in vivo* during the microbial synthesis of phloroglucinol using *E. coli* PG1/pBC2.274 is of particular interest. To this end, a series of 2D protein gel electrophoresis experiments were carried out. Three protein samples were prepared using a 1 L fermentation run of PG1/pBC2.274. The first cell sample (Figure 1, control) was collected at OD_{600} = 50 before induction of phloroglucinol synthase synthesis using IPTG. The fermentation was terminated 6 h post-induction and the cells were harvested. A portion of the cells was washed with HEPES buffer and centrifuged to pellet (Figure 1, cell paste). A third sample was prepared by collecting cell debris after several French Press passages (Figure 1, cell

debris). The remaining cells were used to prepare insoluble protein fraction (Figure 1, inclusion bodies). These samples were loaded on 2D protein gels using a pH gradient ranged from 5.5 to 6.7 and an intense dark spot was observed at the intersection of pH 6 and 38 kDa on all the gels. This protein spot was excised from the gel and analyzed by tendem mass spectroscopy with electrospray ionization method. Using the protein identification software Scafford, the isolated protein fraction was unambiguously identified as phloroglucinol synthase. Therefore, it was concluded that culturing phloroglucinol producing microbe PG1/pBC2.274 led to the formation of a significant amount of inclusion bodies under fermentor-controlled conditions. Attenuate PhlD expression level in *E. coli* might lead to a better phloroglucinol synthesizing biocatalyst by alleviate metabolic burden due to the expression of inactive protein.

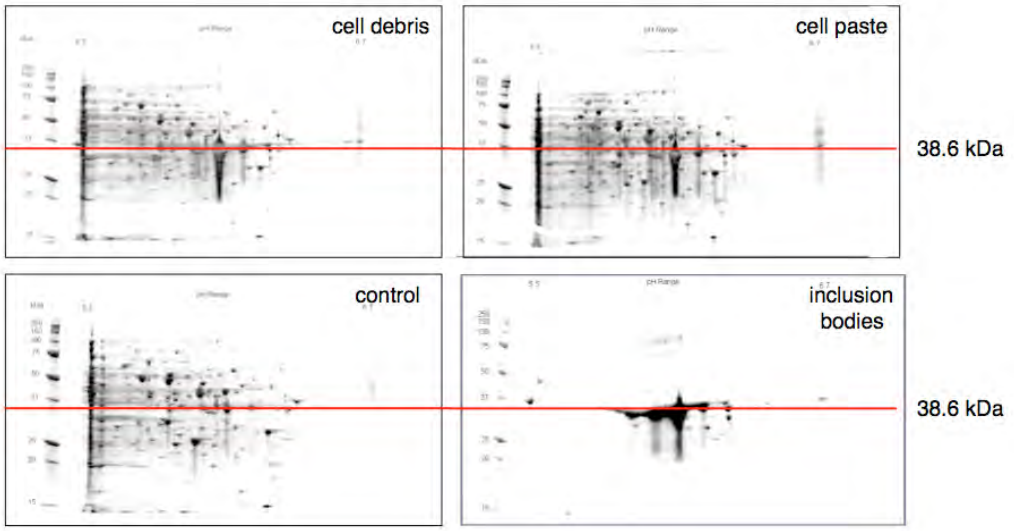

Figure 1. 2D protein gel electrophoresis of PG1/pBC2.274 samples.

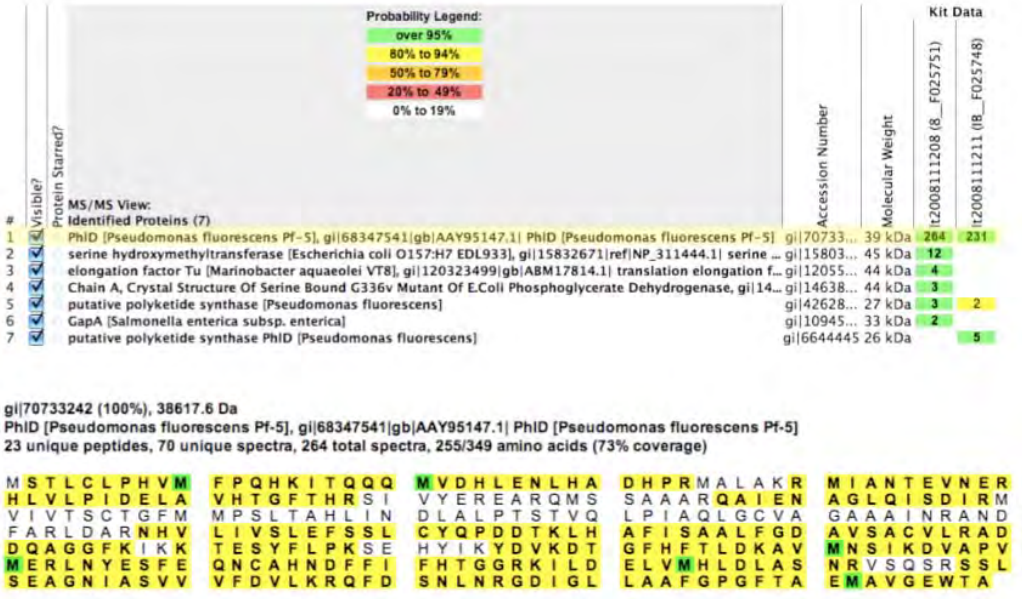

Figure 2. PhlD identification using Scafford.

A series of genetically engineered *E. coli* was developed accordingly. Among these candidates, *E. coli* PG1/pKIT10.080 demonstrated a significant improvement in strain stability and phloroglucinol production under fed-batch fermentor-controlled cultivation. Previously, *in situ*, resin based extractive fermentation was employed to remove toxic substances generated during bacterial culturing. This technology was used once again to remove toxic phloroglucinol from the production tank. Under optimized extractive fermentation conditions, *E. coli* PG1/pKIT10.080 synthesized 25 g/L phloroglucinol in 92 h at a yield of 9% (mol/mol) based on glucose consumed.

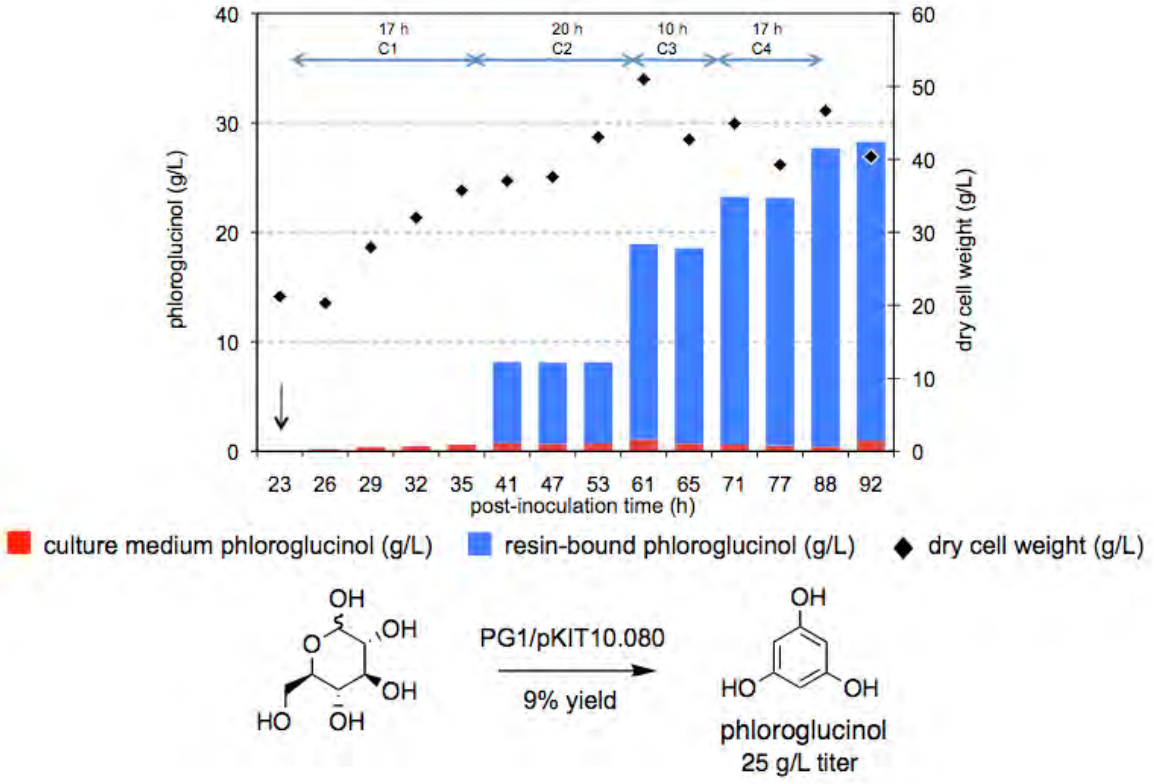

Figure 3. Resin-based extractive fermentation of *E. coli* PG1/pKIT10.080.

Four full length PhlD sequences have been published to date. PhlD from *Pseudomonas fluorescens* Q2-87 (designated as $PhlD^{Q2\text{-}87}$) is 83% identical at the protein sequence level with $PhlD^{Pf\text{-}5}$. PhlD from *Pseudomonas fluorescens* HP72 (designated as $PhlD^{HP72}$) is 85% identical at the protein sequence level with $PhlD^{Pf\text{-}5}$. PhlD from *Pseudomonas fluorescens* 2P24 (designated as $PhlD^{2P24}$) is 84% identical at the protein sequence level with $PhlD^{Pf\text{-}5}$. In addition, approximately 240 partial PhlD sequences are listed in the NCBI GenBank. As with the aforementioned PhlD full length sequences, the partial PhlD sequences are very similar and share 85% or greater sequence identity with $PhlD^{Pf\text{-}5}$. Four phloroglucinol synthesizing *E. coli* PG1/PhlD_Pf5, PG1/PhlD_HP72, PG1/PhlD_2P24 and PG1/PhlD_Q287 were constructed using *de novo* synthesized phloroglucinol synthase candidate genes that were codon-optimized for

expression in *E. coli*. *E. coli* PG1/PhlD_Q287 successfully synthesized 4.3 g/L of phloroglucinol under fed-batch fermentation conditions (Figure 4).

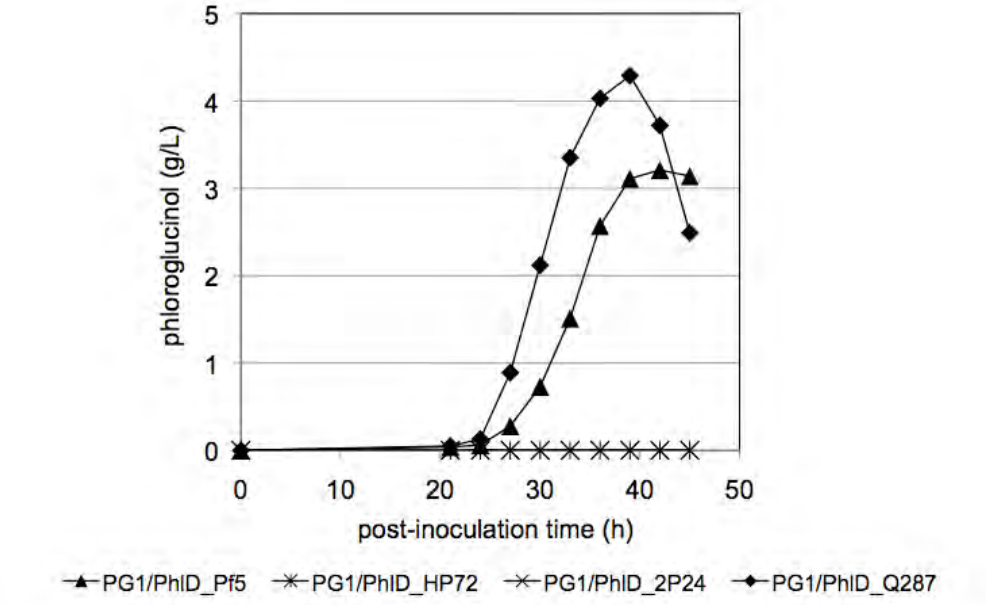

Figure 4. Fed-batch fermentation experiments of PhlD candidates.

All previous phloroglucinol synthases shared approximately 85% identity at the protein sequence level. During the course of our studies, novel phloroglucinol synthases $PhlD^{A}$ and $PhlD^{B}$ were identified that share no more than 46% identity at the protein sequence level with all previously isolated phloroglucinol synthases such as the extensively studied *Pseudomonas fluorescens* Pf-5 phloroglucinol synthase (designated as $PhlD^{Pf\text{-}5}$). Initial identification of these phloroglucinol synthases followed from bioinformatics analyses. The $PhlD^{A}$ protein sequence is only 46% identical with $PhlD^{Pf\text{-}5}$. The $PhlD^{B}$ protein sequence is only 43% identical with $PhlD^{Pf\text{-}5}$. $PhlD^{A}$ and $PhlD^{B}$ are also very different from one another sharing only 46% identity at the protein sequence level. Unlike the *phlACBDE* gene cluster in which $phlD^{Pf\text{-}5}$ resides in *Pseudomonas fluorescens* Pf-5, $phlD^{A}$ and $phlD^{B}$ were not part of a *phlACBDE* biosynthetic gene cluster or any other apparent gene cluster. Genes encoding $PhlD^{A}$ and $PhlD^{B}$ were synthesized and appraised *in vitro* for enzyme activity in the presence of substrate malonylCoA. Crude cell lysate from $PhlD^{A}$ gave specific activity of 0.001 U/mg, while $PhlD^{B}$ gave 0.01 U/mg. Plasmid-localized $phlD^{A}$ and $phlD^{B}$ were also transformed into an *E. coli* host PG1 and the intact constructs evaluated for synthesis of phloroglucinol under fermentor-controlled conditions (Figure 5). Peak phloroglucinol synthesis of 0.2 g/L was observed for *E. coli* PG1/$PhlD^{A}$. This compares with peak phloroglucinol synthesis of 4.0 g/L for *E. coli* PG1/$PhlD^{B}$.

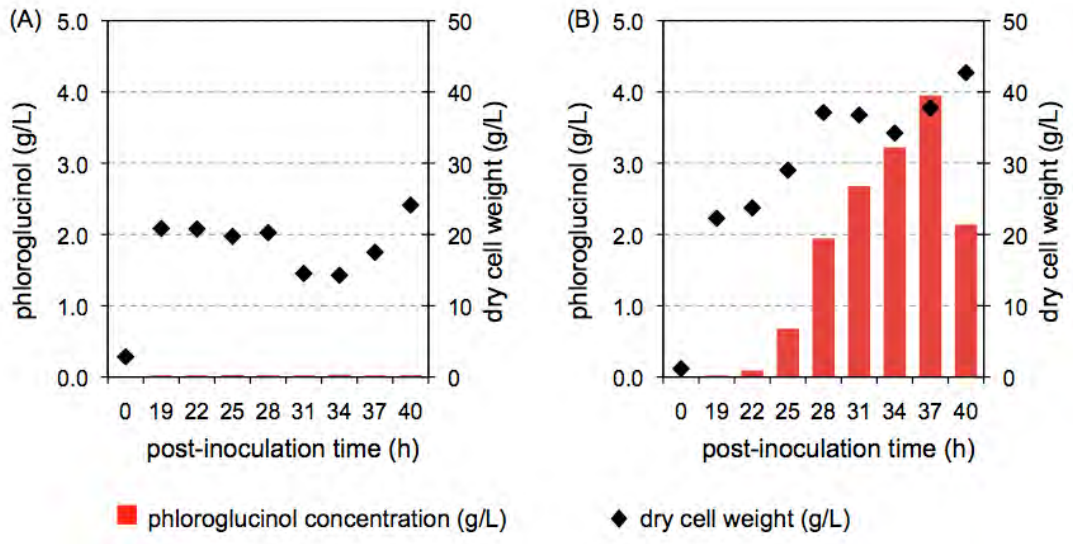

Figure 5. Fed-batch fermentation experiments of *E. coli* PG1/PhlD^{A} and PG1/PhlD^{B}.

In parallel with all these efforts, microbial synthesis of phloroglucinol in *P. fluorescens* was also examined. High cell density cultivation of *P. fluorescens* was achieved in the fermentation vessel using minimal salt medium. The medium formulation is shown in Figure 6. Fed-batch fermentation of wild-type *P. fluorescens* was carried out using this medium formulation and an optical density at 600 nm reached 82 shortly after 36 h cultivation. Cultivation of *P. fluorescens* phloroglucinol synthesizing construct Pf1.162/pJA2.232 under a similar culturing condition produced 0.1 g/L phloroglucinol (Figure 7). This experiment demonstrated the first example of microbial synthesis of phloroglucinol in *P. fluorescens* species under fed-batch conditions using minimal growth medium.

Components	g/L
glucose	15 g
KH_2PO_4	13.3 g
$(NH_4)_2HPO_4$	4.0 g
$MgSO_4 \cdot 7H_2O$	1.2 g
citric acid	1.7 g
EDTA	8.4 mg
$CoCl_2 \cdot 6H_2O$	2.5 mg
$MnCl_2 \cdot 4H_2O$	15.0 mg
$CuCl_2 \cdot 2H_2O$	1.5 mg
H_3BO_3	3.0 mg
$Na_2MoO_4 \cdot 2H_2O$	2.5 mg
$Zn(CH_3CO_2)_2 \cdot 2H_2O$	13.0 mg
Fe(III) citrate	100.0 mg
thiamine•HCl	4.5 mg

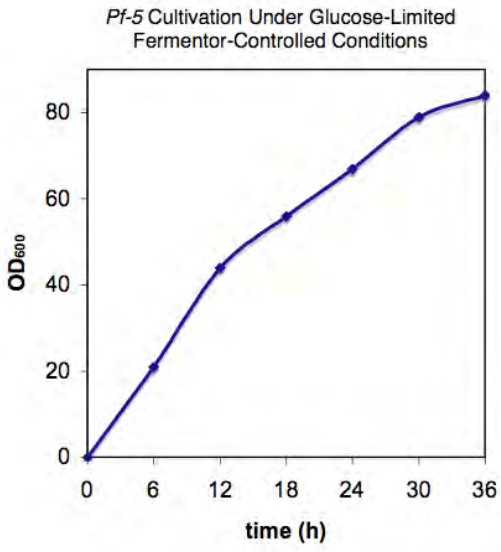

Figure 6. Minimal medium formulation for *P. fluorescens* cultivation.

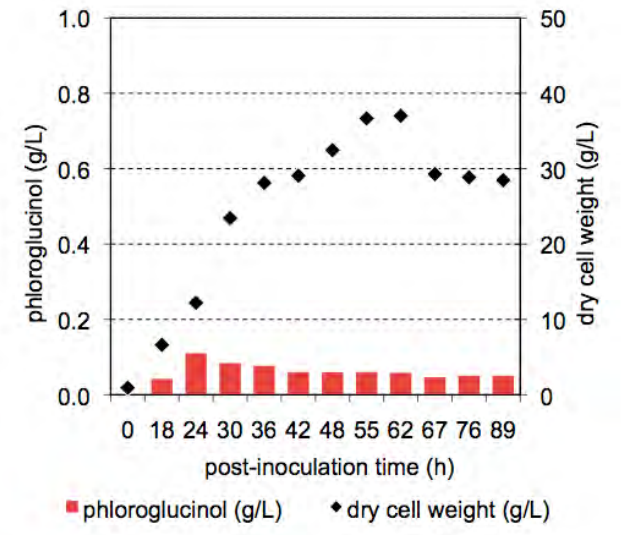

Figure 7. Fed-batch fermentation experiment of *P. fluorescens* Pf1.162/pJA2.232.

e. Work Plan

Efforts will continue to manipulate *phlD* at the genetic level to increase its specific activity and its catalytic lifetime under fermentor-controlled conditions. Prospecting will continue for new sources of phloroglucinol synthase activity. Reaction engineering will be pursued to further optimize fermentor-controlled phloroglucinol production. Metabolic engineering in *Pseudomonas fluorescens* species will be continued. Hopefully, related efforts will allow us to devise an efficient phlroglucinol producing biocatalyst.

f. Major Problems/Issues

None.

g. Technology Transfer

h. Foreign Collaborations and Supported Foreign Nationals